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Human, donkey and cow milk differently affects energy efficiency and inflammatory state by modulating mitochondrial function and gut microbiota☆

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Abstract

Different nutritional components are able, by modulating mitochondrial function and gut microbiota composition, to influence body composition, metabolic homeostasis and inflammatory state. In this study, we aimed to evaluate the effects produced by the supplementation of different milks on energy balance, inflammatory state, oxidative stress and antioxidant/detoxifying enzyme activities and to investigate the role of the mitochondrial efficiency and the gut microbiota in the regulation of metabolic functions in an animal model. We compared the intake of human milk, gold standard for infant nutrition, with equicaloric supplementation of donkey milk, the best substitute for newborns due to its nutritional properties, and cow milk, the primary marketed product. The results showed a hypolipidemic effect produced by donkey and human milk intake in parallel with enhanced mitochondrial activity/proton leakage. Reduced mitochondrial energy efficiency and proinflammatory signals (tumor necrosis factor α, interleukin-1 and lipopolysaccharide levels) were associated with a significant increase of antioxidants (total thiols) and detoxifying enzyme activities (glutathione-*S*-transferase, NADH quinone oxidoreductase) in donkey- and human milk-treated animals. The beneficial effects were attributable, at least in part, to the activation of the nuclear factor erythroid-2-related factor-2 pathway. Moreover, the metabolic benefits induced by human and donkey milk may be related to the modulation of gut microbiota. In fact, milk treatments uniquely affected the proportions of bacterial phyla and genera, and we hypothesized that the increased concentration of fecal butyrate in human and donkey milk-treated rats was related to the improved lipid and glucose metabolism and detoxifying activities.

Keywords: Mitochondria; Microbiota; Milk; Redox-status; SCFAs

1. Introduction

Evidence from animal and human studies indicates that mitochondrial function and gut microbiota influence metabolic homeostasis.

Mitochondria play a central role in cellular function and metabolism by coupling cellular respiration to the production of ATP. However, this coupling is not perfectly tight. Approximately 20% of the standard metabolic rate in mammals is due to a leak of protons across the mitochondrial inner membrane (basal proton leak) in a manner that uncouples cellular respiration from ATP production, thereby generating heat [1]. In addition to this basal leak, there is an inducible leak of protons catalyzed by free fatty acids (FFAs) [2]. Recent work suggests that an inducible proton leak might have an important role in the protection against reactive oxygen species (ROS) [3]. Moreover, mitochondria play a key role in cell signaling through production of ROS that modulate redox signaling. Nuclear factor E2-related factor-2 (Nrf2) is a transcription factor that orchestrates the expression of battery of antioxidant and detoxification genes under both basal and stress condition [4]. Nrf2 also modulates genes involved in metabolic

Abbreviations: ALT, alanine aminotransferase; CM, cow milk; CPT, carnitine-palmitoyl-transferase; DM, donkey milk; FFA, free fatty acid; GSH, glutathione; GSSG, glutathione disulfide; GST, glutathione-*S*-transferase; HM, human milk; HOMA, homeostasis model assessment; IL-1, interleukin-1; IL-10, interleukin-10; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; NQO1, NAD(P)H:Quinone Oxidoreductase; Nrf2, nuclear factor erythroid-2-related factor-2; OTUs, operational taxonomic units; RCR, respiratory control ratio; ROS, reactive oxygen species; SCFAs, short-chain fatty acids; TNF-α, tumor necrosis factor α; UCP2, uncoupling protein-2.

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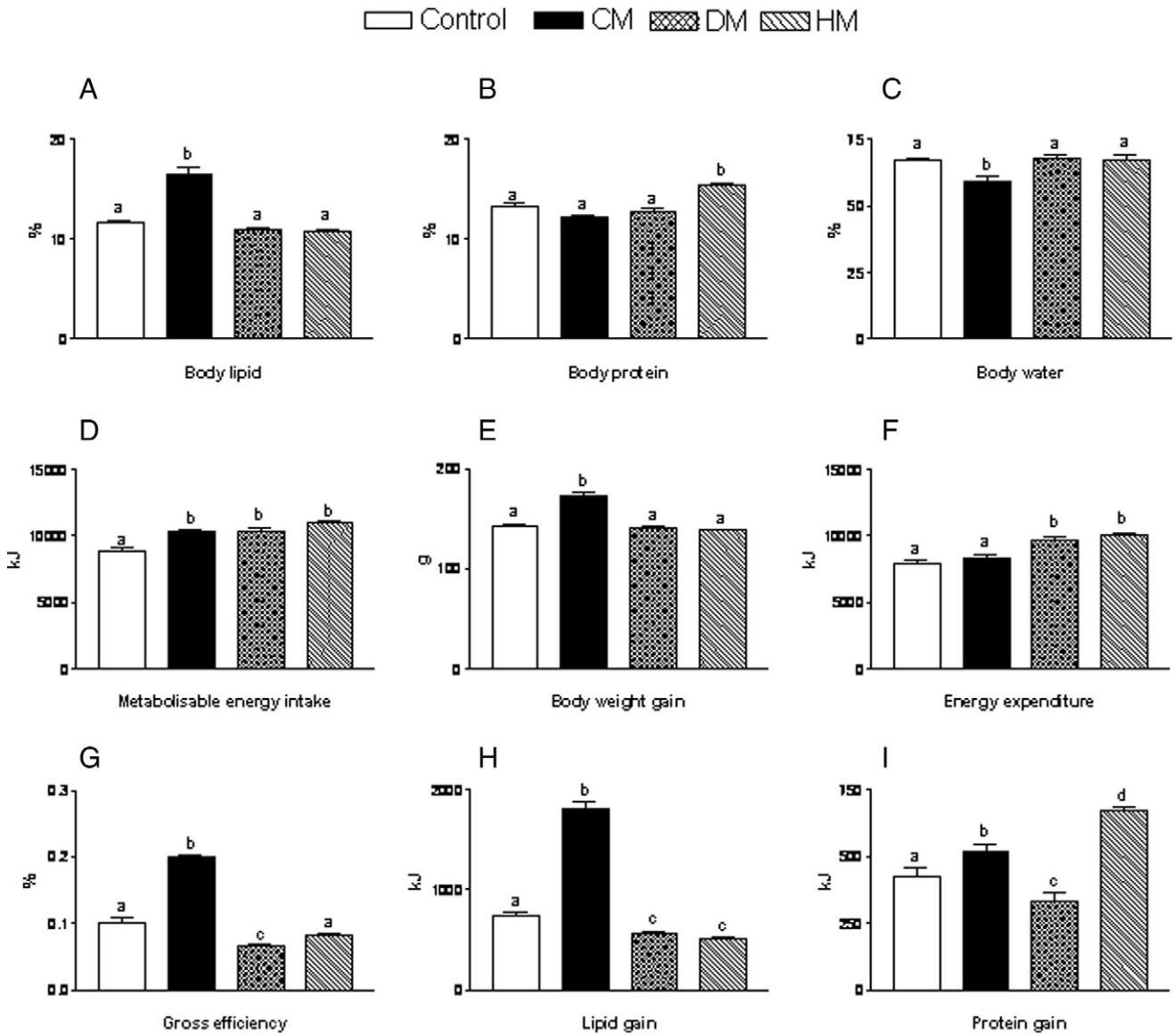


Fig. 1. Body composition and energy balance. Body lipid (A), body protein (B), body water content (C), metabolisable energy intake (D), body weight gain (E), energy expenditure (F), gross efficiency (G), lipid gain (H) and protein gain (I) were reported. The results are expressed as the means±S.E. from $n=7$ animals/group. Different superscripted letters indicate statistically significant differences ($P<.05$).

regulation, such as fibroblast growth factor 21 (FGF21), a liver-derived pro-lipolytic hormone and peroxisome proliferator-activated receptors (PPARs), which play an important role in nutrient homeostasis [5]. Notably, the link between metabolism, ROS homeostasis and mitochondrial metabolism is further indicated by the role played by PPARγ coactivators-1 (PGC-1). Among them, PGC-1α plays an important role in mitochondrial biogenesis and in the regulation of genes responsible for ROS detoxification [6], while PGC-1β is involved in mitochondrial metabolism and its activation exerted a protective effects from lipid overload [7].

Animal and human studies indicate that the metabolic function develops primarily after birth when the newborn is first exposed to nutrition via the gastrointestinal tract and that the composition of the gut microbiota influences body composition, digestion and metabolic homeostasis [8,9].

Phylogenic changes in gut microbiota composition and the production of bacterial metabolites, including short-chain fatty acids (SCFAs), can interact with reach host tissues to act as metabolic regulators and to control energy metabolism and inflammatory state [10].

Therefore, nutritional strategies are able, by influencing the mitochondrial function [11,12] and microbiota composition, to favor the appropriate development of metabolic and immune system functions, and this strategy is now considered a feasible means to prevent diseases such as obesity, diabetes and allergies [8,9].

Table 1
Serum parameters

	Control	CM	DM	HM
Glucose (mg/dl)	138.6±2.1^a	130.1±5.7^a	112.1±2.7^b	112.7±3.7^b
Insulin (μg/l)	0.273±0.018^a	0.274±0.019^a	0.271±0.019^a	0.201±0.013^b
HOMA index	2.19±0.12^a	2.09±0.13^a	1.75±0.12^b	1.314±0.10^c
Triglycerides (mg/dl)	116±5.3^a	132±7.5^a	90±4.1^b	120±5.2^a
Total cholesterol (mg/dl)	61±1.6^a	63±1.1^a	58±1.2^a	59±1.5^a
ALT (U/l)	67±2.0^a	71±3.2^a	62±2.0^a	44±2.6^b

Data are the means ± S.E. Data with different superscripted letters are significantly different ($P<.05$).

Relevance of early nutrition in promoting body growth and health is well established. Human milk (HM) is the natural food of all human infants; it provides an adequate supply of all nutrients necessary to support growth and development and even has a key role in preventing overweight and obesity throughout life in addition to providing immunoregulatory components [13]. Unfortunately, a very large population of infants are deprived of their natural food at a proportion that greatly exceeds the possible deficiencies of their mothers. The rate of cow milk (CM) consumption in the first months of life is high in Western countries, which has been suggested as a potential factor contributing to the increasing burden of obesity and related disorders [13]. The overall proportion of nutrients contained in CM, such as protein content and quality, fatty acid profile, iron level and the near absence of nondigestible carbohydrates, may pose health concerns for children aged <1 year [14]. Moreover, CM intake in the first months of life has raised concerns because of its association with allergies [15].

Consistently during recent years, milks from monogastric animals, rather than from ruminant species, have been indicated to be more suitable for human nutrition based on their physicochemical properties. Among these, donkey milk (DM) has been suggested as the best potential substitute for HM due to its composition [16]. Moreover, as a result of its remarkable nutritional value coupled with its reduced allergenicity and good palatability, DM has been suggested as a replacement diet for infants affected by CM allergies when HM is not available [17].

Comparative data on the metabolic effects of DM and CM, using HM as the reference standard, are still scarce. In this study, we aimed to evaluate the differently ability of DM, CM and HM, by modulating mitochondrial function and gut microbiota, to affect energy balance, lipid and glucose metabolism, inflammatory and oxidative stress in animal model.

2. Materials and methods

Male Wistar rats (Charles River, Calco, Lecco, Italy) were individually caged in a temperature-controlled room and exposed to a daily 12- to 12-h light–dark cycle with free access to chow and drinking water. Young animals (60 days old) (average weight, 345 g) were used; one group ($n=7$) was sacrificed at the beginning of the study to establish baseline measurements. The remaining rats were divided into four experimental groups ($n=7$): three groups were supplemented with equicaloric intake (82 kJ) of raw CM, DM or HM (21, 48 or 22 ml/day, respectively) for 4 weeks; the group that did not receive milk supplement was used as control. Despite the different volumes used, the energy density provided by the different milk supplements was comparable (energetic composition of diets are reported in Supplementary Table S1 and S2).

At the end of the treatments, the animals were anesthetized by intraperitoneal injection of chloral hydrate (40 mg/100 g body weight), and blood was taken from the inferior cava and portal vein. The liver was removed and subdivided; samples not immediately used for mitochondrial preparation were frozen and stored at −80°C.

All experiments were conducted in compliance with national guidelines for the care and use of research animals (D.L. 116/92, implementation of EEC directive 609/86).

2.1. Evaluation of body composition and energy balance

During the treatments, the body weights and food intake were monitored daily to calculate weight gain and gross energy intake. Spilled food and feces were collected daily for precise food intake calculation. Energy balance assessments were conducted over the 4 weeks of treatment by the comparative carcass evaluation [18]. Metabolizable energy intake was obtained by subtracting the energy measured in the feces and urine from the gross energy intake, which was determined from the daily food consumption and gross energy density. The gross energy density for the standard diet, CM, DM or HM (15.8, 14.04, 13.79 or 14.01 kJ/g, respectively) as well as the energy density of the feces and the carcasses were determined by bomb calorimetry (Parr adiabatic calorimeter; Parr Instrument Co., Moline, IL, USA). The evaluation of the energy, fat and protein content in animal carcasses was conducted according to a published protocol [18]. Energy efficiency was calculated as the percentage of body energy retained per metabolizable energy intake, and energy expenditure was determined as the difference between metabolizable energy intake and energy gain.

Table 2
Immunomodulatory markers

	Control	CM	DM	HM
TNF-α (ng/ml)	0.092±0.006[a]	0.090±0.005[a]	0.055±0.005[b]	0.058±0.005[b]
MCP-1 (ng/ml)	3.62±0.15[a]	3.82±0.22[a]	3.29±0.22[a]	3.87±0.32[a]
IL-1 (pg/ml)	56.4±3.3[a]	64.0±2.1[a]	42.9±3.4[b]	42.2±3.9[b]
IL-10 (ng/ml)	0.061±0.003[a]	0.134±0.008[b]	0.177±0.012[c]	0.200±0.005[d]
LPS (EU/ml)	0.704±0.028[a]	0.668±0.025[a]	0.584±0.014[b]	0.580±0.015[b]

Data are the means±S.E. Data with different superscripted letters are significantly different ($P<.05$).

2.2. Mitochondrial parameters

Mitochondrial isolation, oxygen consumption and proton leakage measurements were performed as previously reported [19]. Oxygen consumption (polarographically measured using a Clark-type electrode) was measured in the presence of substrates and ADP (state 3) and in the presence of substrates alone (state 4), and their ratio (respiratory control ratio, or RCR) was calculated. The rate of mitochondrial fatty acid oxidation was assessed in the presence of palmitoyl-L-carnitine. Mitochondrial proton leakage was assessed by a titration of the steady-state respiration rate as a function of the mitochondrial membrane potential in liver mitochondria. This titration curve is an indirect measurement of proton leakage because the steady-state oxygen consumption rate (i.e., proton efflux rate) in nonphosphorylating mitochondria is equivalent to the proton influx rate due to proton leakage. The CPT system and aconitase activity were measured spectrophotometrically [20,21]. The rate of mitochondrial H_2O_2 release was assayed by following the linear increase in fluorescence due to the oxidation of homovanillic acid in the presence of horseradish peroxidase [22].

2.3. Liver lipid content, redox state and Nrf2-activated enzyme activities

Total hepatic lipid content was estimated by using the Folch method [23]. Total thiols [(glutathione (GSH)+glutathione disulfide (GSSG)] in plasma and the GSH and GSSG concentrations in the liver were measured with the dithionitrobenzoic acid–GSSG reductase recycling assay [24]; the GSH/GSSG ratio was used as an oxidative stress marker. The enzymatic activities of GST and NQO1 were evaluated spectrophotometrically in liver cytoplasmic extracts with standard protocols [25–27].

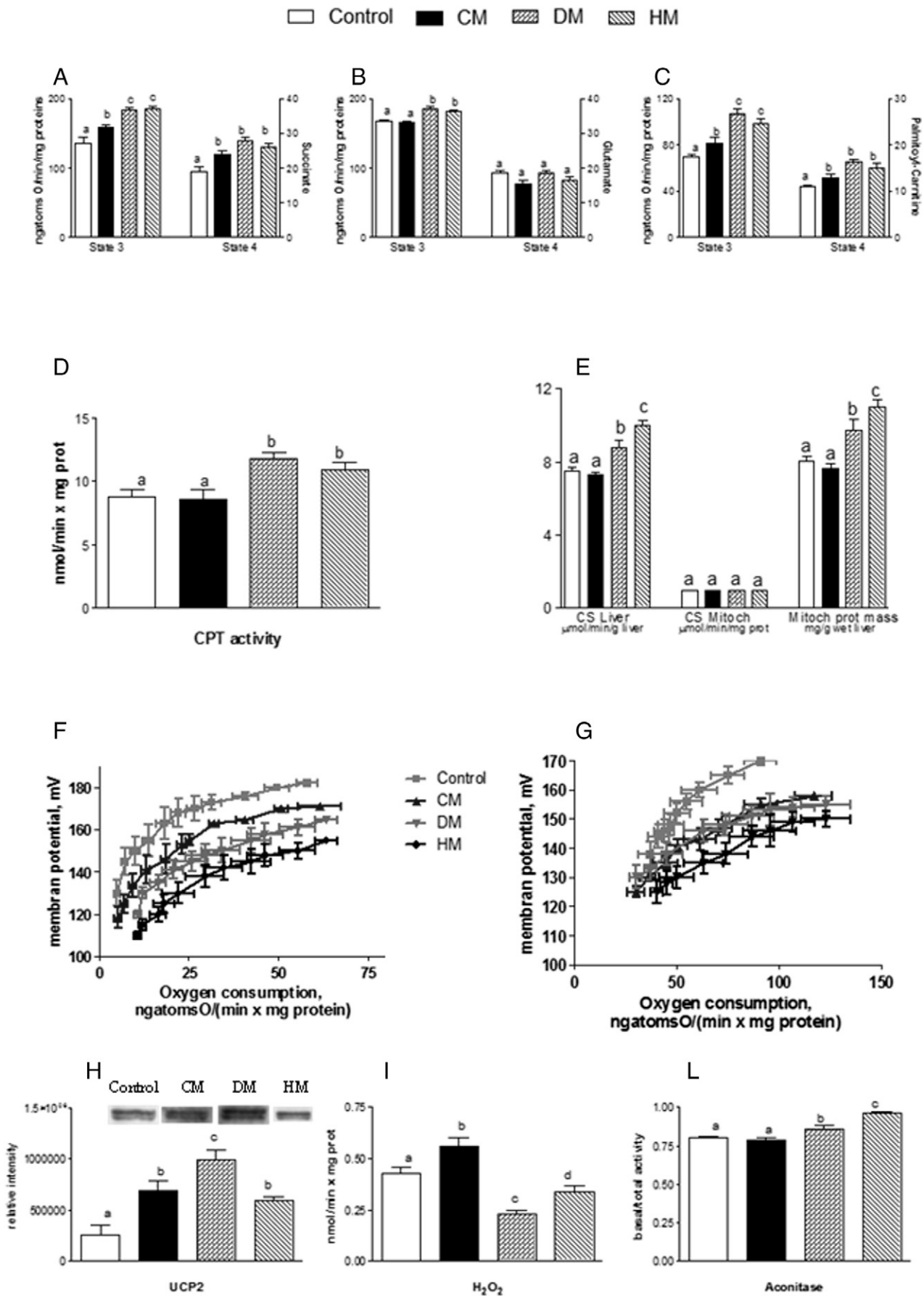
2.4. Quantitative real-time polymerase chain reaction analysis

Total RNA was extracted from liver of rats fed with the different milk (CM, DM, HM) and of control rats using the TRIzol Reagent (Ambion). After DNase treatment (Ambion), RNA was quantified using a Nanodrop 2000c spectrophotometer (Thermo-Scientific) and reverse-transcribed (1 μg) using the Advantage RT-PCR kit (Clontech) and oligo dT primer. Universal Probe Library Assay Design Center (https://www.roche-applied-cience.com/sis/rtpcr/upl/index.jsp?id=UP030000) was used for designing primers (Supplementary Table S3). The Real-Time PCR reactions were performed using a 7500 Real-Time PCR System (Applied Biosystems) in the presence of 1× Power Sybr Green PCR Master mix (Applied Biosystems) and 0.1 μM of each primer and 30 ng of cDNA. The thermal protocol was as follows: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. For all of the genes examined, the reactions were conducted in technical duplicates. For each well, the evaluation of PCR efficiency (E) and optimal threshold cycle (CT) of the target genes (*PGC1α*, *PGC1β*, *PPARα*, *PPARβ*, *FGF21*) and the endogenous control gene (β-actin) were performed using the REAL TIME PCR MINER online tool [28]. The mean relative expression ratio (rER) of the target genes was calculated using β-actin as the endogenous control gene and cDNA of the control rats as the reference sample applying the formula:

$$\mathrm{rER} = (1+\mathrm{E\ target\ gene})^{-\Delta \mathrm{CT\ target\ gene}} / (1+\mathrm{E\ endogenous\ control})^{-\Delta \mathrm{CT\ endogenous\ control}},$$

where ΔCT target gene is the difference between the CT value of the target gene in the liver of the CM, DM, HM rats and the CT value of the target gene in the liver of the control rats, $\Delta CT_{\text{endogenous control}}$ is the difference between the CT value of the β-actin gene in

Fig. 2. Coupling efficiency in the liver mitochondria. Liver mitochondrial respiration rates were evaluated in the presence of succinate (A), glutamate (B) or palmitoyl-carnitine (C) substrates. Carnitine-palmitoyl transferase (CPT) activity was reported (D). Citrate synthase (CS) activity was measured in the liver homogenate and mitochondrial fractions; the mitochondrial protein mass was calculated as the ratio between CS activity in the homogenate and isolated mitochondria (E). Basal (F) and fatty acid-induced proton-leakage (G) was measured in liver mitochondria. Representative immunoblot of UCP2 expression in liver mitochondria; the bands were quantified by densitometry. The values are expressed as relative intensity in arbitrary units (H). Intracellular H_2O_2 yield (I) and the basal aconitase/total aconitase ratio are reported (L). The results are expressed as the means±S.E. from $n=7$ animals/group. Different superscripted letters indicate statistically significant differences ($P<.05$).


Control
CM
DM
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A
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Oxygen consumption,
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H
relative intensity
UCP2
I
nmol/min x mg prot
H2O2
L
basal/total activity
Aconitase

liver of the CM, DM, HM rats and the CT value of the β-actin gene in the liver of the control rats [29].

2.5. Preparation of fecal extracts

Feces were collected and were frozen at −20°C. Frozen feces were diluted with saline solution and centrifuged at 13,000 rpm for 10 min. Supernatants were filtered (0.45 μM) and used as the fecal extracts. These extracts were stored at −20°C until further analysis.

2.6. Quantification of fecal SCFAs concentrations

Frozen fecal extracts were acidified with 20 μl 85% phosphoric acid and 0.5 ml ethyl acetate. The contents were mixed by vortexing, centrifuged at 14,000 rpm for 1 h and extracted in duplicate. A quantity of the pooled extract containing the acidified SCFAs was transferred into a 2-ml glass vial and loaded onto an Agilent technologies 7890 gas chromatography system with an automatic loader/injector. The GC column was an Agilent J&W DB-FFAP (Agilent Technologies, Leini, TO, Italy) of length 30 m, internal diameter 0.25 mm and film thickness 0.25 μm. The GC was programmed to achieve the following run parameters: initial temperature 90°C, hold 0.5 min, ramp 20°C/min, final temperature 190°C, and total run time 8.0 min. Gas flow was set at 7.7 ml/min splitless to maintain 3.26 psi column head pressure with a septum purge of 2.0 ml/min. Detection was achieved using a flame ionisation detector. Peaks were identified using a mixed external standard and quantified by the peak height/internal standard ratio.

2.7. Microbiota analysis: DNA isolation and sequencing

The cecal contents of rats collected postmortem were stored at −80°C. Metagenomic DNA was extracted from the cecal contents using a QIAamp-DNA stool mini-kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and the adapted procedure described by Dewulf et al. [30]. High-throughput sequencing of metagenomic samples was performed by DNAVision (Gosselies, Belgium). Briefly, the V1–V3 region of the bacterial 16S rRNA gene was amplified using barcoded primers 27f (5′-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG-3′) and 534r (5′-ATTACCGCGGCTGCTGG-3′) [31], and purified amplicons were analyzed on a Roche FLX Genome Sequencer using titanium chemistry. The resulting Q25 reads were processed through the QIIME v1.7.0 pipeline [32]. Sequences were depleted of barcodes and primers, sequences <200 bp or >1000 bp were removed, sequences with ambiguous base calls were removed, and sequences with homopolymer runs exceeding 6 bp were removed. The sequences were then de-noised, and chimeras were removed. Operational taxonomic units (OTUs) were defined by clustering at 3% divergence (97% similarity). The OTUs were then taxonomically classified using BLASTn against a curated GreenGenes database.

2.8. Statistical analyses

Data are presented as the means±S.E. unless otherwise indicated. Differences among groups were compared by analysis of variance followed by the Newman–Keuls test to correct for multiple comparisons. Differences were considered statistically significant at $P<.05$. Analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

For microbiota analyses, data are expressed as the means±S.D. Differences between groups were assessed using a two-tailed Student's *t* test. In the case of nondetectable values for some samples, a nonparametric test was required, such as Fisher's Exact Test to compare two groups at a time. Because most of the parameters had an abnormal distribution (assessed using a Shapiro normality test), the correlations were analyzed by Spearman's correlation. Data were analyzed using JMP 8.0.1 (SAS Institute, Inc., Cary, NC, USA), R 3.0.2 (The R Foundation) and RStudio 0.97.310 with the package gplots for the heatmap. The results were considered statistically significant at $P<.05$.

3. Results

3.1. Body composition and energy balance

Although different milk treatments provided similar metabolizable energy intake (Fig. 1D), we found that CM-treated animals exhibited an increase in body weight gain (Fig. 1E) and body lipids percentage (Fig. 1A) compared to control, whereas the same parameters of DM- or HM-treated animals were similar to control. A significant increase in body protein content was observed in HM-treated rats (Fig. 1B). In DM and HM groups, we observed an increased energy expenditure (Fig. 1F). Finally, the gross energy efficiency was significantly increased in CM-treated and significantly decreased in DM-treated animals compared to control animals. This parameter was not affected in HM-treated animals (Fig. 1G). The enhanced energy efficiency in CM-treated animals was associated with higher lipid gain (Fig. 1H). In contrast, this parameter was markedly reduced in DM- and HM-treated animals compared to controls. Protein gain (Fig. 1I) was significantly decreased in DM-treated animals and increased in the other two groups, compared to control. In particular, HM-treated animals showed a greater increase compared to the control.

3.2. Serum metabolites and inflammatory parameters

Serum glucose levels were significantly lower in HM- and DM-fed rats than in the other animals, HM-fed rats showed the lowest insulin concentration among the groups. Consequently, a marked reduction of homeostasis model assessment (HOMA) index was observed in the DM and HM groups compared to controls, with HM producing the lowest HOMA index (Table 1). Total cholesterol levels were not affected by the different milks; however, triglycerides were significantly decreased in DM-fed animals and alanine aminotransferase (ALT) was significantly decreased in HM-fed animals (Table 1). Tumor necrosis factor α (TNF-α), interleukin (IL)-1 and lipopolysaccharide (LPS) concentrations were significantly decreased in HM- and DM-fed animals compared to control and CM-fed rats, whereas monocyte chemoattractant protein-1 (MCP-1) levels resulted unaffected by the different milks. IL-10 was increased by two to three-fold in the treatment groups (HM>DM>CM) compared to control (Table 2).

3.3. Mitochondrial efficiency and oxidative stress

Mitochondrial state 3 respiration using succinate or palmitoylcarnitine (fatty acid oxidation) as a substrate was increased in CM-treated animals and further increased in DM- and HM-treated animals compared to controls (Fig. 2A–C). State 3 respiration using glutamate (Fig. 2B) as substrate and CPT activity (Fig. 2D) were increased in DM- and HM-treated animals compared to control and CM-treated rats. RCR values were indicative of high-quality mitochondrial preparations (data not shown).

After 4 weeks of treatment, the specific activity of citrate synthase in isolated mitochondria was unchanged, whereas in liver homogenates, the activity was higher in DM-treated animals and further increased in HM-treated animals compared to control and CM-treated rats (Fig. 2E). Therefore, a significantly higher mitochondrial protein contents (calculated as the ratio between citrate synthase activity in the homogenate and isolated mitochondria) found in HM- and DM-treated rats (HM>DM) indicate that the improved oxidative capacity appears to be supported (at least in part) by an increased mitochondrial mass (Fig. 2E). Mitochondrial basal and FFA-induced proton leakage was increased in milk-treated animals (Fig. 2F–G) compared to control. The effect on mitochondria basal leakage was related to the type of milk used (HM>DM>CM) (Fig. 2F). In particular, HM-treated animals exhibited the highest oxygen consumption to maintain the same membrane potential among the groups. In FFA-induced proton-leakage conditions, mitochondria from DM- and CM-treated rats exhibited comparable kinetic curves (Fig. 2G). UCP2 significantly increased after milk treatments, but this effect was more pronounced in DM-treated animals (Fig. 2H).

Next, H_2O_2 yield and ROS-induced damage were measured in mitochondria. H_2O_2 yield was significantly increased in CM-treated rats and was reduced in mitochondria from the HM and DM groups (HM>DM) (Fig. 2I). Aconitase activity was significantly increased in DM and HM-treated rats compared to CM and control rats (HM>DM) (Fig. 2L).

3.4. Antioxidant/detoxifying defences

Antioxidant state and cytoprotective enzyme activities are improved by dietary supplementation with DM or HM. We

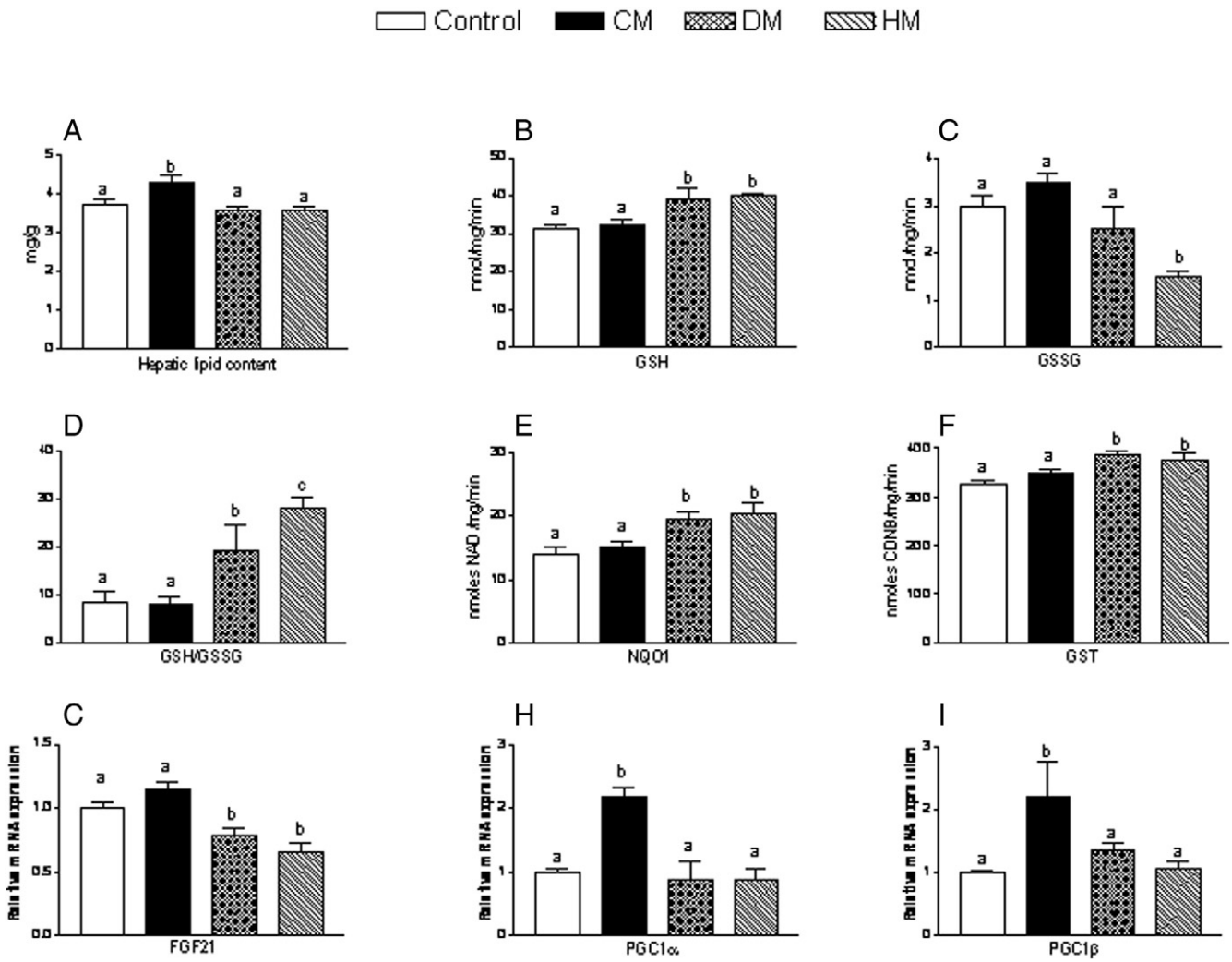

Fig. 3. Redox status and antioxidant/detoxifying defences. Total lipid (A), GSH (B) and GSSG content (C), GSH/GSSG ratio (D), GST (E) NQO1 activity (F), relative mRNA expression of FGF21 (G), PGC1α (H), and PGC1β (I) were measured in the livers of differently treated rats. The results are expressed as the means±S.E. from $n=7$ animals/group. Different superscripted letters indicate statistically significant differences ($P<.05$).

measured the total protein thiols in the serum, we found a two-fold increase in total GSH levels in the sera from HM-treated animals (0.199±0.008 nmol/mg-protein/min) compared to controls (0.101 ±0.003); to a lesser extent, we also found an increase of total GSH in CM- and DM-supplemented rats (0.128±0.002 and 0.154±0.014 nmol/mg-protein/min). At hepatic level, CM-treated rats exhibited a significantly higher percentage of lipids compared to other groups (Fig. 3A). Reduced GSH levels were significantly increased in animals supplemented with DM or HM compared to CM-treated or control rats (HM=DM>CM=C) (Fig. 3B). Moreover, the beneficial effects produced by the HM and DM treatments on liver redox status were clearly indicated by the marked decline in the GSSG content and the significantly increased GSH/GSSG ratios (Fig. 3C–D). In addition, the activity of NQO1 and GST was significantly higher in DM- or HM-treated rats compared to CM-treated or control rats (Fig. 3E-F). In addition, because Nrf2 activation was demonstrated to induce the expression of metabolic genes (*PPARs*, *FGF21*) [5], we analyzed the expression of the *PPARα*, *PPARγ*, *PGC1α*, *PGC1β* and *FGF21* genes. No variation was observed in the expression levels of *PPARα*, *PPARγ* (data not shown). The *FGF21* mRNA levels in DM and HM were significantly decreased compared with CM rats (Fig. 3G). The CM rats tended to increase the *FGF21* mRNA levels in the liver, compared with control. However, this difference was not statistically significant (Fig. 3G). *PGC1-α and PGC1-β* mRNA levels significantly increased in CM compared to the other groups (Fig. 3H–I).

3.5. Gut microbiota composition

Gut microbiota composition was analyzed by pyrosequencing. Bacteroidetes was increased in CM-fed rats (+22%) and Verrucomicrobia was decreased in DM-fed rats (−58%) compared to control. TM7 was increased (+575%) in DM-fed rats compared to CM-fed rats (Fig. 4). Data on phylum differences are provided in Supplementary Table S4.

The abundances of 10 genera were significantly affected by milk treatments (Fig. 5). DM and HM similarly modified gut microbiota at genera level (Supplementary Table S5).

The potential link between changes in gut microbiota composition and host metabolic parameters following different milk treatments was evaluated by a Spearman correlation coefficient (Fig. 6). *Streptococcus* and *Lactococcus* genera (increased in DM) and *Coprobacillus* and *Parabacteroides* (increased in HM) were negatively correlated with inflammatory markers (LPS and TNF-α) and fasting blood glucose (Fig. 6). Moreover, these genera were positively correlated

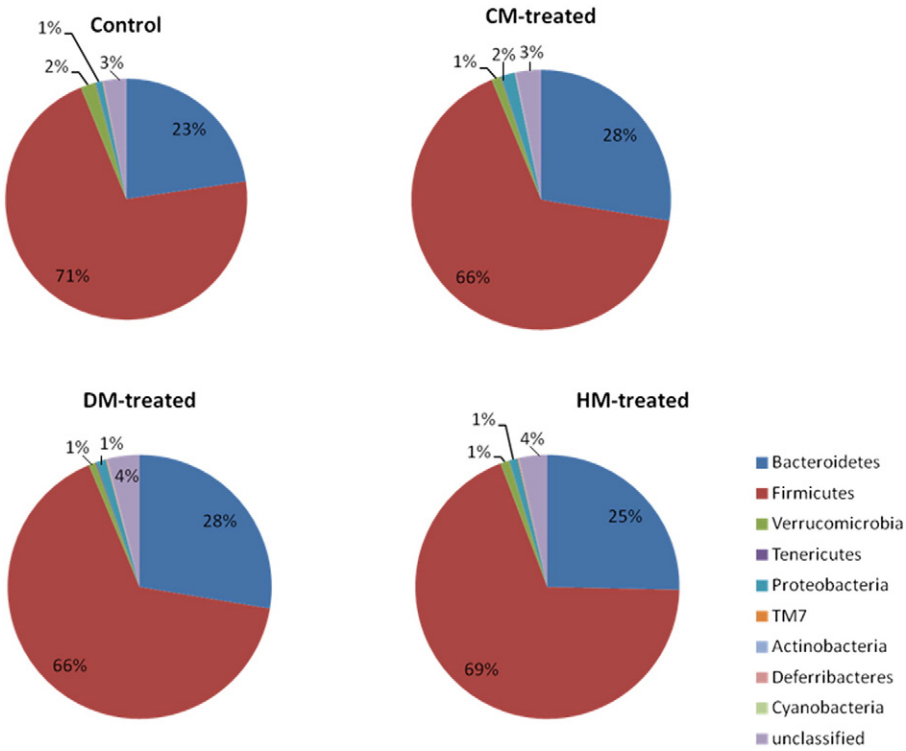

Fig. 4. Milk treatments affect the proportions of different phyla. The composition of abundant bacterial phyla identified in gut microbiota of control and different milk-treated animals. Undetected phyla are not represented on the pie chart. The significant differences in specific phyla are shown in Supplementary Table 4.

with fecal butyrate content, mitochondrial respiratory capacity and fatty acid oxidation rate (Fig. 6). In contrast, *Akkermansia* abundance was positively correlated with fasting blood glucose, TNF-α and LPS levels. Changes in *Blautia* and *Syntrophococcus* genera were both positively correlated with antioxidant and anti-inflammatory markers (serum GSH and IL-10). *Blautia* abundance (increased by all milks) was positively correlated with fecal butyrate, mitochondrial respiratory capacity and fatty acid oxidation rate. In addition, the significant increase of *Syntrophococcus* by HM (Supplementary Table S5) was positively correlated with energy expenditure and body protein content (Fig. 6).

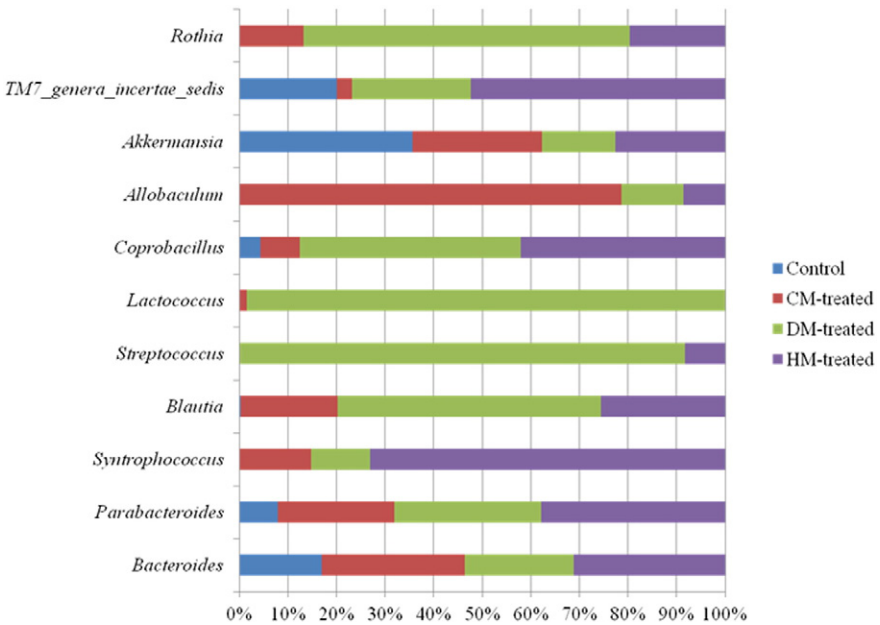

Fig. 5. Milk treatments affect the proportions of different genera. The composition of the bacterial genera significantly affected by the treatments and identified in gut microbiota of control and different milk-treated animals. Each bar is set at 100% to illustrate the proportion of each genera among the different groups; the absence of any color indicates that the genus was not detected in this group of rats. Statistically significant changes observed between different groups are shown in Supplementary Table 5.

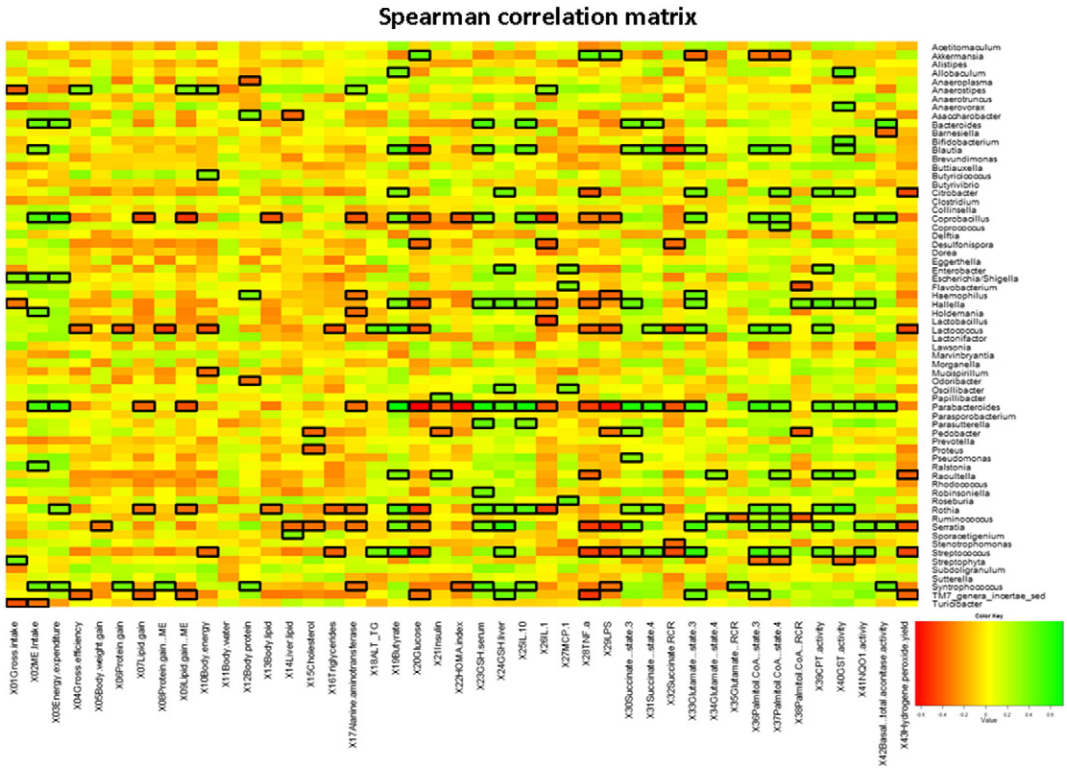

Fig. 6. Spearman correlations heat map Heat map of the Spearman r correlations between the gut bacteria modified by the different milk supplements and biological parameters. Correlations were performed on values for each rat in every group.

3.6. SCFAs production

An increase in fecal butyrate and propionate levels was observed in animals treated with DM or HM, but not CM, compared with control rats. The effect was two-fold greater in DM-treated compared with HM-treated animals (Fig. 7).

4. Discussion

In this study, we compared the nutritional, immunomodulatory and antioxidant effects of iso-energetic supplementation with milk from three distinct animals: two that are monogastric (HM and DM) and one that is polygastric (CM). We recently showed the efficacy of DM in decreasing the accumulation of body lipids and its ability to improve the use of fat as metabolic fuel for hepatic mitochondria [33]. Here, we demonstrate that these biological effects are comparable with those elicited by HM. Although these different milks resulted in similar increases in metabolizable energy, DM and HM had no effect on body weight gain but were able to increase energy expenditure compared with CM. The enhanced energy efficiency found in CM-fed animals was associated with higher total body and liver lipid levels; in contrast, these values were markedly reduced in DM-and HM-fed animals. In HM-fed rats, it is likely that the decrease in adiposity can be explained by increased fatty acid oxidation; however, we cannot exclude that this effect may also be due to lower plasma insulin levels. The roles that essential fatty acids, enzymes, hormones, growth factors and other biologically active compounds play in the effects of HM and DM on lipid metabolism merit further consideration. The observed differences in body protein content suggest some interesting perspectives. The quality of proteins, with respect to their essential amino acid content, may be responsible for the difference observed. However, we cannot rule out that this effect on protein gain may also be due to the high concentration of growth factors in HM that are essential for the proliferation and differentiation of body tissues [34].

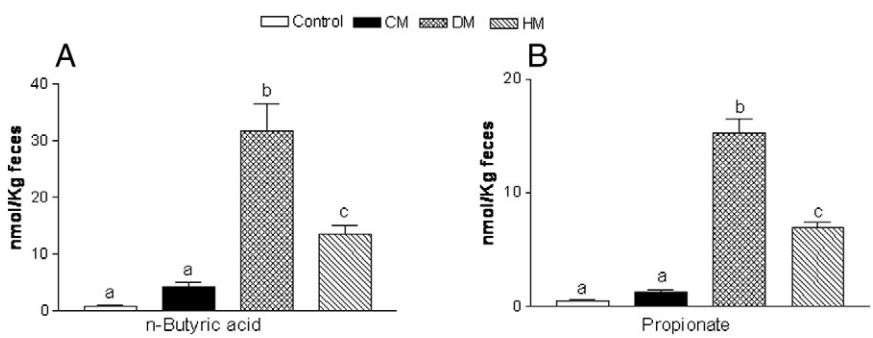

Fig. 7. SCFAs concentrations fecal butyrate (A) and propionate (B) levels were reported. The results are expressed as the means±S.E. from $n=7$ animals/group. Different superscripted letters indicate statistically significant differences ($P<.05$).

The current study highlights the ability of HM to control glucose homeostasis by inducing a dampened glycemic response compared to CM and the lowest insulin levels and HOMA index compared to other milks. These results are consistent with Gunnerud et al. [35]. Other data show that the insulin response to milk is not only due to the lactose component but is also related to the whey fraction [36] and to bioactive peptides in the milk or secreted in the gastrointestinal tract [37]. Moreover, glucose-regulating hormones, such as leptin, adiponectin, resistin and ghrelin, detected in HM [38], may be involved in the regulation of growth in infancy and might influence the programming of energy balance with long-term consequences on health [13]. Other studies highlight the correlation between low blood glucose and insulin in infancy and low insulin levels in adulthood, suggesting a protective role of breastfeeding against type 2 diabetes and obesity in later life [39]. It is noteworthy that in the rat model, DM affects glucose metabolism in a manner more similar to HM than CM, suggesting that in addition to providing a hypoallergenic milk substitute for children affected by CM protein allergies, DM might have beneficial effects by changing energy homeostasis in favor of fatty acid oxidation, thereby reducing fat storage. However, this speculation warrants further investigation in humans. Moreover, the metabolic benefits on body weight and glucose control induced by HM and DM may be related to the modulation of the gut microbiota. We and others have previously shown that the gut microbiota plays a major role in energy storage and glucose homeostasis [40,41]. Among the metabolites produced by the gut microbiota, SCFAs have been shown to play a major role [42,43]. We can speculate that the marked increase in SCFAs in both DM- and HM-treated rats can be responsible, at least in part, to beneficial effects found in these animals. Finally, the effects of SCFAs have been described in several studies and have been shown to reduce diet-induced obesity and to increase energy expenditure [42–44].

The effect of different milks on nutritional status is controlled by multiple mechanisms. Considering the central role of the liver in energy expenditure and lipid and glucose metabolism, we evaluated hepatic mitochondrial function and efficiency.

We observed similar biological responses in rats fed with DM or HM; in particular, the beneficial effects of DM administration are further enhanced in rats treated with HM. HM-fed rats exhibited increased respiratory capacity and mitochondrial mass and decreased oxidative stress in their liver mitochondria even when the ability to utilize fat as a metabolic fuel was increased. The increased mitochondrial fatty acid oxidation observed in the liver is likely related to an enhancement of CPT activity, which would further increase the entry of long-chain FFAs into the mitochondria. The consequent increase in lipid oxidation is apparently sufficient to handle the decreased load of hepatic FFAs and body adiposity.

A concomitant decline in mitochondrial energy efficiency (thermogenic effect), as evidenced by the increased proton-leakage and increased UCP2 protein content in HM-fed rats, may also contribute to burning the fat in these animals. Indeed, in addition to stimulating fatty acid oxidation in the liver, HM induced a less efficient utilization of lipid substrates through the stimulation of a thermogenic mechanism, such as proton leakage. We speculate that HM-fed animals (similarly to DM-treated rats) might be protected from the development of obesity through this mechanism; however, this hypothesis requires further investigation. In addition, the reduction of mitochondrial oxidative stress parameters (H_2O_2 production and aconitase activity) can result from the concomitant increase in proton leakage, which was reported as a major mechanism involved in the modulation of membrane potential to control mitochondrial ROS emission [3]. The observed beneficial effects elicited by DM intake on the antioxidant status (GSH/GSSG ratio) and on detoxifying enzyme activities (GST-NQO1) confirm previous findings [33]. Notably, the comparable enhancement of antioxidant/detoxifying defences by DM and HM intake is attributable, at least in part, to the activation of the Nrf2–ARE pathway and further supports the feasibility of DM as an ideal HM substitute. Moreover, Nrf2 may exert a control on the stress hormones FGF21 [45], an important factor in the homeostatic mechanisms regulating glucose and lipid metabolism [46]. Importantly, the activation of Nrf2 in DM and HM rats is significantly associated with lower mRNA expression levels of FGF21, whereas the increased FGF21 expression in CM group if compared to DM or HM rats, together with high hepatic lipid content and H_2O_2 yield, is in line with the expression of PGC1 that in previous studies was showed necessary for the FGF21 protective effects in liver damage conditions, regulates ROS homeostasis and mitochondrial biogenesis [47].

Moreover, due to the recognition of the gut–liver axis and on the basis of the recent studies reporting the *in vivo* and *in vitro* ability of butyrate to modulate the Nrf2–ARE pathway [48,49], our data showing increased concentrations of fecal butyrate in HM- and DM-treated rats led us to hypothesize that an improved Nrf2 defence may result from the increased levels of butyrate-producing microbiota in these animals. Finally, the reported association of enhanced anti-inflammatory defences with Nrf2 signaling [50] prompted us to evaluate the consequences of DM and HM intake on the levels of several proinflammatory indicators. The beneficial effects produced by HM and DM intake were indicated by lower TNF-α and IL-1 concentrations, decreased LPS levels (marker of metabolic and inflammatory diseases) [44] and increased IL-10.

Here, we investigated the impact of CM, DM and HM dietary supplementation on the gut microbiota composition. We found that DM and HM affected the gut microbiota in favor of two genera that have been linked with anti-inflammatory properties: *Bacteroides* and *Parabacteroides*. Importantly, these bacterial species are known to produce SCFAs [51]. It is worth noting that we found an increased level of fecal SCFAs in animals fed with DM and HM. Recent evidence suggests that the genus *Parabacteroides* may be associated with an improved intestinal integrity and a lower inflammatory tone [52]. In agreement, we also found (by Spearman correlation) that *Parabacteroides* abundance was negatively associated with several inflammatory markers. Moreover, an increase of *Syntrophococcus* and *Blautia* genera in milk-treated rats was observed. Interestingly, these two genera were correlated with different biological parameters. For instance, *Syntrophococcus*, which was increased in the HM-treated group, is positively related to body protein content and gain, whereas *Blautia*, which was significantly increased in the DM-treated group, is positively associated with mitochondrial respiratory capacity. It is worth noting that in patients affected by hepatic encephalopathy, the presence of *Blautia* was associated with good cognition, lower severity of liver disease and decreased inflammation [53]. We did not find any differences among the groups in *Lactobacillus* genera; however, we observed a significantly higher rate of *Streptococcus* and *Lactococcus* in DM-treated rats than in other groups. It was previously shown that a probiotic combination containing *Streptococcus thermophilus* protects the bowel and improves colonic inflammation in experimentally induced inflammatory bowel disease in rats [54]. Our findings support this hypothesis because *Streptococcus* and *Lactococcus* were negatively correlated with inflammatory parameters (TNF-α, LPS and H_2O_2 yield). We also analyzed *Akkermansia* rate. This species was isolated from the human intestinal tract due to its efficient use of mucus as a carbon and nitrogen source, and it is associated with the protective mucosal lining of the intestine. We and others have previously shown that *Akkermansia muciniphila* abundance is correlated with an improved metabolic profile [55,56]. In addition, it was demonstrated that *A. muciniphila* treatment reversed high fat diet-induced metabolic disorders by restoring the gut mucus layer that is disrupted with obesity and type 2 diabetes [55]. It is likely that *A. muciniphila* regulates gut barrier function at different levels. Excessive mucin degradation by intestinal bacteria may contribute to inflammatory

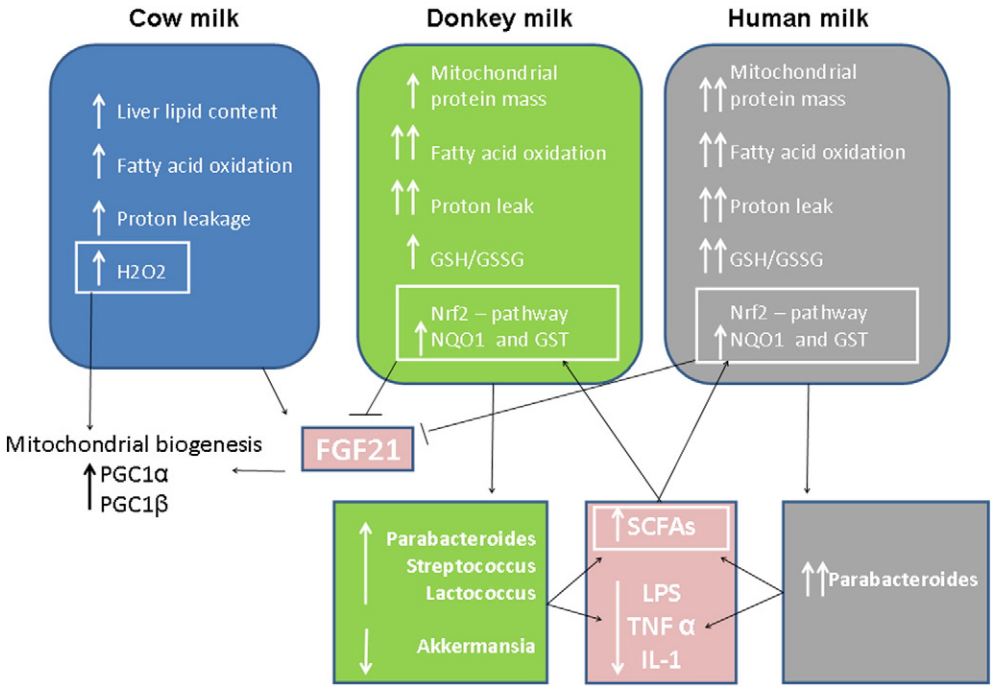

Fig. 8. Model depicting the effects of distinct milks supplementation on, lipid and glucose metabolism, inflammatory and oxidative stress by modulating mitochondrial function and gut microbiota. To assess the effects of distinct milks supplementation on energy balance and redox state, it is crucial to understand how mitochondrial function and gut microbiota composition interact with host and participate in the metabolic response to diet. The modulation of Nrf2 pathway and the bacterial metabolism of nutrients in the gut, with the release of bioactive compounds (SCFAs), promote competitive interactions with host cellular targets to control energy metabolism and inflammatory state. Nrf2 pathway is induced by DM and HM and leads to a decrease in FGF21 expression. In contrast, in CM rats, the lacking activation of Nrf2 abolishes its inhibitory effect on FGF21 modulating mitochondrial biogenesis by PGC1. DM and HM affect gut microbiota composition with an increase in SCFAs which in turn modulate Nrf2. The correlation between gut microbiota and inflammatory parameters is confirmed by Spearman analyses.

bowel diseases because the access of luminal antigens to the intestinal immune system is facilitated, whereas in pathological conditions, *A. muciniphila* seems to improve intestinal response by increasing mucus production and the number of goblet cells [55]. In this study, no increase of *Akkermansia* was found in the treated groups; instead a significant reduction of its presence was observed in DM-treated rats and was positively correlated with fasting glucose, TNF-α and LPS levels. Whether the beneficial effects of the different milks observed in our study are mediated through this genus warrants further investigation.

The present study highlights that dietary supplementation with HM or DM is associated with a decrease of inflammatory status. This decrease is associated with the improvement of lipid and glucose metabolism. In addition, our observations indicate that the beneficial effects elicited by HM and DM are, at least in part, mediated by their ability to modulate mitochondrial function and efficiency, ROS homeostasis and Nrf2–FGF21 pathways. Moreover, we found that specific gut microbes and metabolites (SCFAs) were increased upon HM and DM feeding, thereby linking gut microbiota with host metabolism (Fig. 8).

Altogether, our study adds further support to the exceptional qualities of HM and DM compared to CM. Thus, the impact of milk from ruminants differs from that of monogastric species on gut microbes and host metabolism at different levels (energy storage, energy expenditure, mitochondrial function, metabolism and inflammation). Whether the physicochemical and nutritional properties can explain the distinctive nutritional, sensory and metabolic characteristics among the different milks requires further studies. Finally, nature provided humans, just as all mammalian species, with a milk especially suited not only to the nutritional needs of the respective infants but also to the promotion of future health and development. Nevertheless, by displaying novel mechanisms linking gut microbes and mitochondrial function with cellular metabolic responses according to the milk used, this study adds novel significant perspectives and suggests that selected milks may provide protection against specific metabolic disorders.

Conflict of interest statement

The authors have declared no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2015.05.003.

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